Spatio-temporal expression patterns of (*E*)-β-farnesene-binding protein genes in the turnip aphid, *Lipaphis erysimi* (Hemiptera: Aphididae)

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Abstract: 【Aim】 Odorant binding proteins (OBPs) contribute to the remarkable sensitivity of insect olfactory system and play an important role in aphid alarm pheromone (*E*)-β-farnesene (EBF) communication. The turnip aphid, *Lipaphis erysimi* (Kaltenbach) is the major pest of cruciferous crops. To reduce pesticide application in vegetable fields, EBF appears to hold strong potential for aphid control. However, few studies about *L. erysimi* to EBF are known so far. 【Methods】 Two putative OBP genes, *LeryOBP*3 and *LeryOBP*7 encoding proteins with high affinity to EBF were cloned by PCR and RACE and characterized from *L. erysimi*, and their expression profiles at different developmental stages and in different tissues of wingless adults of *L. erysimi* were determined by RT-qPCR. 【Results】 The two genes cloned here, *LeryOBP*3 (GenBank no.: KJ703012) and *LeryOBP*7 (GenBank no.: KJ703013) share high amino acid sequence identities (94% and 88%, respectively) with *ApisOBP*3 and *ApisOBP*7, which encode proteins with affinity to EBF in *Acyrthosiphum pisum* (Harris). The full length of open reading frames of *LeryOBP*3 and *LeryOBP*7 are 357 and 468 bp, encoding 118 and 155 amino acids, respectively. Developmental expression profiles showed that the obvious expression peaks of *LeryOBP*3 and *LeryOBP*7 appeared at the adult stage, and tissue expression profiles showed that *LeryOBP*3 was expressed strongly in legs of adults while *LeryOBP*7 in adult antennae. 【Conclusion】 These results suggest that LeryOBP7 in the antennae might be responsible for the response of *L. erysimi* to EBF.

Key words: *Lipaphis erysimi*; alarm pheromone; odorant-binding protein; gene expression profile; real-time quantitative PCR

1 INTRODUCTION

Upon attacked by predator or parasitoid, individual aphid emits alarm pheromone to warn the colony in this danger. When other aphids detect this cue, they remove their stylets from the host plant and fall, jump, or walk away to escape potential danger (Edwards *et al.*, 1973; Pickett *et al.*, 1992; Braendle and Weisser, 2001; Vandermoten *et al.*, 2012). Alarm pheromones are emitted by nearly all aphid species, and the sesquiterpene (*E*)-β-farnesene (EBF) was identified as the primary component of the alarm pheromone for most economically important aphid species (Bowers *et al.*, 1972; Xiangyu *et al.*, 2002). EBF is the only volatile compound identified in the alarm pheromone of 13 aphid species (Francis *et al.*, 2005). For other aphid species, the production of EBF is often accompanied by other minor components (Nishino *et al.*, 1977; Pickett and Griffiths, 1980).

Insect antenna is the main olfactory organ, where two olfactory protein families, the odorant binding proteins (OBPs) and the odorant receptors (ORs), are responsible for receiving, transporting and triggering responses to semiochemicals (Zhou *et al.*, 2004; Northey *et al.*, 2016).

OBPs are crucial constituents of insect olfaction systems, and they are considered to solubilize and transport target odor molecules across the lymph to reach the odorant receptors (ORs) (Steinbrecht, 1998; Vogt, 2003; Lartigue *et al.*, 2003; Zhang *et al.*, 2011; Kaissling, 2013; Venthur *et al.*, 2014). For hydrophobic odors such as aphid alarm pheromone, *in vitro* binding studies have provided evidence that OBPs play an important role in ligand capturing and transporting to achieve the ligand OR interaction (Venthur *et al.*, 2014).

The OBP sequences are highly similar among

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different aphid species, including the aphids *Acyrthosiphon pisum* (Harris), with 15 putative OBPs identified through its genome sequence (Zhou *et al.*, 2010b), and *Aphis gossypii* (Glover), with 10 OBPs identified using a high-throughput sequencing platform (Gu *et al.*, 2013). Depending on *in vitro* binding studies, it has been demonstrated that two OBPs of *A. pisum*, ApisOBP3 and ApisOBP7 may play important roles in aphid alarm pheromone discrimination (Sun *et al.*, 2012; Venthur *et al.*, 2014; Northey *et al.*, 2016).

The turnip aphid, *Lipaphis erysimi* (Kaltenbach) is the major pest of cruciferous crops, such as Chinese cabbage *Brassica rapa pekinensis* which is the primary vegetable crop in northern China. To reduce pesticide application in vegetable fields and also to increase vegetable quality and food safety, the aphid alarm pheromone appears to hold strong potential for controlling a wide variety of aphid pests (Cui *et al.*, 2012a, 2012b). It was reported that EBF, which was isolated from *Matricaria chamomilla* L. (Asterales: Asteraceae) essential oil, released in Chinese cabbage fields, could synergize the influence of insecticide to improve the control of *Myzus persicae* (Sulzer) and *L. erysimi* (Cui *et al.*, 2012a). So we hypothesized that both *M. persicae* and *L. erysimi* could respond to the EBF isolated from *M. chamomilla*. Although much attention has been paid to the olfactory behavior of *M. persicae* to EBF and EBF-biding proteins (Edwards *et al.*, 1973; Sun *et al.*, 2012; Sun *et al.*, 2013; Verheggen *et al.*, 2013), few studies about *L. erysimi* to EBF are known so far.

It was reported that the behavioral responses to synthetic EBF exhibited by some cruciferous aphids, such as *L. erysimi* and *Brevicoryne brassicae* (L.), were weaker compared with most other EBF-producing aphid species (Dawson *et al.*, 1987). However, the specific OBP3 putatively combined with EBF in *B. brassicae* was characterized (Fan, 2011). Comparing with *M. persicae* and *B. brassicae* which usually infested on the same cruciferous host plants with *L. erysimi* in temperate region, we supposed that EBF-binding proteins that have conserved regions in aphids might be involved in the olfactory response of *L. erysimi*, which also prompted us to clone and characterize OBP genes encoding proteins probably with specific affinity to EBF from *L. erysimi*. So RT-qPCR was adopted to study the expression patterns of *OBP*3 and *OBP*7 at different developmental stages and in different adult tissues of *L. erysimi*. This study could provide an important step towards understanding the behavioral responses of cruciferous aphids and the molecular basis of chemosensory binding to EBF so as to improve the aphid control tactics in terms of push-pull strategy in the cruciferous crop fields.

2 MATERIALS AND METHODS

2.1 Insect rearing

L. erysimi adult aphids were collected from turnip fields in autumn at Tai'an (117°09′E, 36°09′N), Shandong Province, China, and reared on turnip *Brassica rapa* subsp. *rapa* in an environmental chamber operated at 20 ± 2℃, under a photoperiod of 16L∶8D. After generations, the nymphs at different instars and the wingless adults were collected for the experiment.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted using PureLink RNA Mini Kit (Ambion, USA) according to the manufacturer's protocol. The integrity of the total RNA was examined using 2% agarose electrophoresis, and the purity was determined by the ratio of A_{260}/A_{280} measured by a spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA). After measuring the total RNA, 1 μg RNA was subjected to reverse transcription (RT) into first-strand cDNA by TransScript II First-Strand Synthesis SuperMix (Trans, China).

2.3 Gene cloning and sequencing

To clone the cDNA sequences encoding LeryOBP3 and LeryOBP7, the primers designed from regions of OBP3 and OBP7 that are conserved in aphids were used (Table 1). PCRs were performed on a TaKaRa PCR machine (TP600, TaKaRa Bio Inc., Japan) using the EasyPfu PCR SuperMix (Trans, China) with antennal cDNA as a template. The amplification conditions were 5 min at 94℃, followed by 35 cycles of 30 s at 94℃, 30 s at 50℃, 40 s at 72℃, and final extension for 10 min at 72℃. PCR products were separated by electrophoresis on 2% agarose gels in 1 × TAE buffer. The specific fragments were cut and purified

Table 1　Primer list for cDNA cloning

Genes	Primers	Primer sequences (5′-3′)
*LeryOBP*3	OBP3_DEG1_F	ACCATATGCGATGATTTCGTCGACGTTTTAC
	OBP3_DEG1_R	RTRCGANTWRYTGCACGTCGCNAY
*LeryOBP*7	OBP7_DEG1_F	ATGGTCGCCCGGAAAAGAATGTATAAC
	OBP7_DEG1_R	CTAGAGTGGTAGAAACTCTAAACTTTTG

by QIAquick gel extraction kit (Qiagen, Germany) following the manufacture's protocol. Amplified products were cloned into the pEASY BLUNT clone vector (Trans, China). After transformation of *Escherichia coli* DH5α competent cells with the ligation products, positive colonies were selected by PCR using the plasmid primers M13F and M13R and incubated in LB/ampicillin medium. Plasmids were sequenced at BioMad Company (Beijing, China).

2.4 RACE and RT-PCR

The preparation of 5′ and 3′ RACE-ready cDNA and PCR reactions of target genes were conducted following the instructions of the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA). Single strand cDNA was synthesized with Revert Aid™ First-Strand cDNA Synthesis Kit (Fermentas, Canada). After obtaining the full-length gene sequences, normal RT-PCR was performed with each specific primer pair (Table 2) using rTaq DNA polymerase (Takara Bio Inc., Shiga, Japan). The resulting amplified products were subjected to electrophoresis on 1% (w/v) agarose gels in TBE and visualized with ethidium bromide.

Table 2 Specific primer pair list for RT-PCR

Genes	Purpose	Primers	Primer sequences (5′-3′)
LeryOBP3	Total length	OBP3 _F	CGATTTACGACGGAGCAAATCGATT
		OBP3 _R	TCAAGTTGACTTGTCGAGATCCAAAAG
LeryOBP7	5′RACE	OBP7 _5inner	CTTCACTCAAGTAAGCGTCGCAATC
		OBP7 _5outer	TTGGAGCATACGGTTTTCAACATCT

2.5 Sequence analysis

Complete coding sequences of target genes were compiled from the three sections of cDNA fragments (5′, 3′ and core sequence). Open reading frames (ORFs) were identified using the ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). By using the NCBI BLAST network server, protein sequences were identified and amino acid sequences of the same family in the same or different species were found and retrieved from GenBank. We used BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to perform homology searches and ClustalX to align the amino acid sequences.

2.6 RT-qPCR analysis of expression profiles of OBP genes

Total RNA was extracted from healthy individual nymph at different instars including the 1st, 2nd, 3rd, and 4th instars and adult, and from different tissues including antenna, head, thorax, leg, abdomen, embryo of healthy wingless adults of *L. erysimi*, using PureLink RNA Mini Kit (Ambion, USA). Each treatment was replicated three times (50 aphids per replication). First-strand cDNA was synthesized using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Trans, China). The 18S rRNA and *L27* genes were used as the internal control. The RT-qPCR primers (Table 3), and 18S-F/18S-R, l27-F/l27-R sharing similar Tm values, were designed to amplify 100-250 bp fragments. RT-qPCR was performed on an ABI 7500 Real-Time System (Applied Biosystems, USA). Amplifications were carried out in a volume of 20 μL containing 10 μL 2 × SYBR Green Real-time PCR Mix (Toyobo, Japan), 1 μL of 10 μmol/L primers, 8 μL nuclease-free water and 1 μL of each cDNA template. PCR amplification was performed in triplicate wells, using the cycling parameters: 94℃ for 5 min, followed by 40 cycles of 5 s at 94℃, 10 s at 60℃ and 15 s at 72℃. Each relative transcript level was calculated using the comparative $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). For the comparative $2^{-\Delta\Delta C_T}$ method, the amplification efficiencies of the target gene and internal control were approximately equal. To confirm this, a pilot trial was carried out to validate the efficiency of each primer by constructing a standard curve.

Table 3 Primers used in RT-qPCR analysis

Genes	Primers	Primer sequences(5′-3′)
LeryOBP3	OBP3_qF	CATCCTCGCCGTAGTCAAATCC
	OBP3 _qR	GTGTTGTTGTATGAACCATCGTCG
LeryOBP7	OBP7_qF	TCCTCACCATAATAATAGCAGCGAC
	OBP7_qR	GGTCTTGGAGCATACGGATTTCA
18S rRNA	18S-F	TCATCAGCTCGCGTTGATTAC
	18S-R	CGTCCACTCCGAAGACCTCA
L27	L27-F	CCGAAAAGCTGTCATAATGAAGAC
	L27-R	GGTGAAACCTTGTCTACTGTTACATCTTG

To further study the function of identified LeryOBP genes we examined the expression profiles of identified OBP genes with RT-qPCR and compared the relative transcript levels of each OBP gene using 18S rRNA and *L27* as double reference genes. The absolute values of the slope of all lines from template dilution plots (log cDNA dilution *vs*

$\triangle C_T$) were close to zero (data not shown). Therefore, the efficiencies of the target and reference genes were similar in our analysis, and the $\triangle\triangle C_T$ calculation method could be used for the relative quantification.

2.7 Statistical analysis

The statistical analysis was carried out with SPSS software (version 15.0) for Windows. The data in terms of relative mRNA were expressed as mean ± standard deviation (*SD*) and were subjected to Tukey's HSD test after one-way analysis of variance (ANOVA).

3 RESULTS

3.1 Cloning of *LeryOBPs*

With the help of genome annotations for pea aphid (*A. pisum*) OBPs and the BLAST tools at NCBI (http://www.ncbi.nlm.nih.gov/), we designed primers and cloned the corresponding OBP genes from the antennae of *L. erysimi* adults. Full-length sequences were obtained by 3′ and 5′ RACE-PCR of segments. According to the BLAST analysis using GenBank, two OBPs shared high amino acid sequence identities of 94% and 86%, respectively, with ApisOBP3 and ApisOBP7 of *A. pisum*, and were named *LeryOBP*3 (GenBank no.: KJ703012) and *LeryOBP*7 (GenBank no.: KJ703013), respectively. The two OBP mature genes possessed ORFs of 357 and 468 bp, encoding 118 aa and 155 aa, respectively. Besides, the alignment result showed a highly conserved six-cysteine feature at the same position in each sequence (Fig. 1).

3.2 Relative expression levels of the two OBP genes at different developmental stages

The expression levels of *LeryOBPs* at different developmental stages were determined by RT-qPCR (Fig. 2). The expression level of *LeryOBP*3 was comparatively high in the 1st instar nymph, decreased at the 2nd instar, and remained unchanged at the 3rd instar and the 4th instar. An obvious expression peak appeared at the adult stage. The expression level of *LeryOBP*7 was also comparatively high in the 1st instar nymphal stage, and decreased from the 2nd instar to the 3rd instar nymph. The expression level of *LeryOBP*7 increased from the 4th instar, and peaked at the wingless adult stage.

3.3 Relative expression levels and tissue distribution of two OBP genes

RT-qPCR experiments were first performed to determine the tissue expression pattern of two OBP genes (Fig. 3). The results showed that the OBP3 and OBP7 genes were expressed in all the tissues of *L. erysimi* wingless adults. *LeryOBP*3 was expressed strongly in legs, comparatively high in antenna, embryo and head, while poorly in thorax and abdomen. *LeryOBP*7 had the highest expression in antenna, but showed negligible expression in the other tissues tested.

4 DISCUSSION

Sequence analysis showed that the key structural features of *LeryOBP*3 and *LeryOBP*7 were consistent with *ApisOBP*3 and *ApisOBP*7 (Qiao *et al.*, 2009; Sun and Song, 2011). The first 3D structure of OBPs from aphids, *Megoura viciae* (MvicOBP3) and *Nasonovia ribisnigri* (NribOBP3) were presented (Northey *et al.*, 2016). The results provided strong evidence for the conclusion that OBP genes were very old and differentiation of different functions of OBPs was earlier than that of aphid species (Zhou *et al.*, 2010a). Thus, our results suggest that the two proteins LeryOBP3 and LeryOBP7 might function together to participate in the sense of ambient EBF in turnip aphid.

We performed RT-qPCR to determine the expression levels of the identified OBP genes in various adult tissues, confirming that *LeryOBP*3 and *LeryOBP*7 were expressed in whole tissues of *L. erysimi*. Both OBP genes were expressed in olfactory related tissues, especially in antenna and legs. The expression of *LeryOBP* in antennae may indicate the presence of these OBPs in olfactory sensilla located in some parts of the antennae (Ban *et al.*, 2014). Particularly, the highest expression level of *LeryOBP*3 in legs (Fig. 3) indicated that the legs of *L. erysimi* could hold the largest number of LeryOBP3-specific olfactory sensilla, suggesting the other function of LeryOBP3 that plays a role in chemical cue identification on plant surface. While the *LeryOBP*7 gene in *L. erysimi* was antenna-specific, which was different from *LeryOBP*3, indicating that LeryOBP7 and LeryOBP3 may have different binding affinities to EBF.

In addition, the highest relative expression levels of *LeryOBP*3 and *LeryOBP*7 appeared in adult stage (Fig. 2). Although the amount of EBF released in cornicle droplets was not higher in asexual adult stage than in nymphal stage (Mondor *et al.*, 2000), the adults could fly or drop away from the dangerous location immediately when they sensed EBF plus glucosinolates. Then the aphids can locate a new habitat to produce next generation as soon as possible to keep their populations. So we inferred that the highest relative expression levels of *LeryOBP*3 and *LeryOBP*7 in adult stage of *L. erysimi* could increase the population fitness in fields.

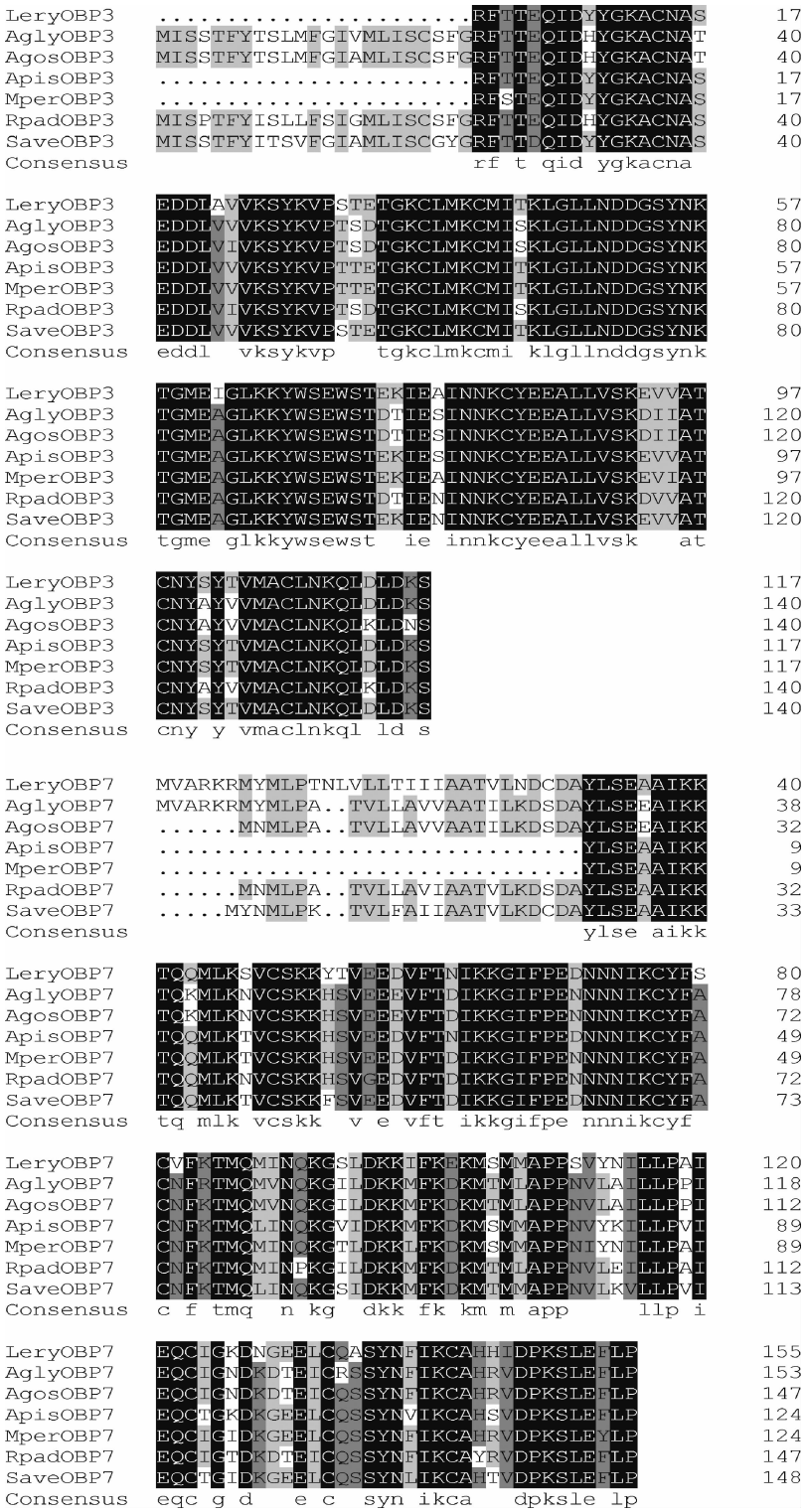

Fig. 1 Alignment of amino acid sequences of OBP3 and OBP7 in aphids

Origin of OBP proteins and their GenBank accession numbers: *Lipaphis erysimi* (Kaltenbach), LeryOBP3 (KJ703012), LeryOBP7 (KJ703013); *Aphis glycines* Matsumura, AglyOBP3 (AHJ80889.1), AglyOBP7 (AHJ80893.1); *Aphis gossypii* (Glover), AgosOBP3 (AGE97633.1), AgosOBP7 (AGE97637.1); *Acyrthosiphon pisum* (Harris), ApisOBP3 (CAR85630.1), ApisOBP7 (CAR85634.1); *Myzus persicae* (Sulzer), MperOBP3 (CAR85644.1), MperOBP7 (CAR85647.1); *Rhopalosiphum padi* (L.), RpadOBP3 (AHL30242.1), RpadOBP7 (AHL30243.1); *Sitobion avenae* (Fabricius), SaveOBP3 (AEX65668.1), SaveOBP7 (ACW03675.2).

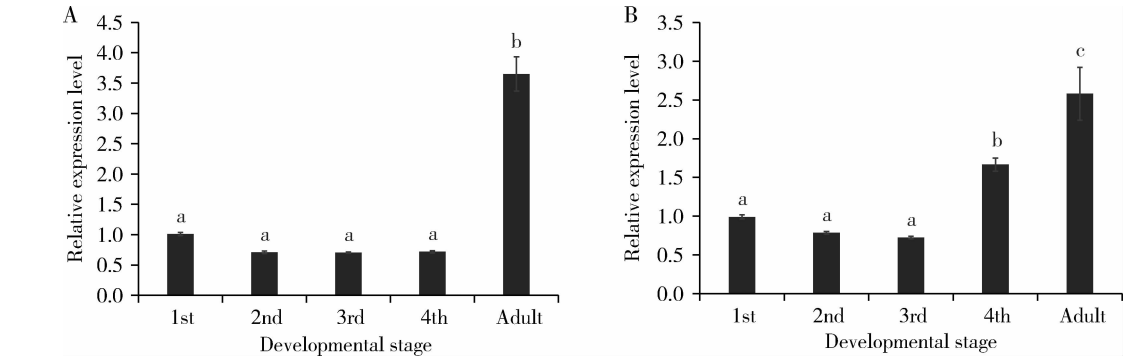

Fig. 2　Relative expression levels of *LeryOBP*3 (A) and *LeryOBP*7 (B) at different developmental stages of *Lipaphis erysimi*
1st: 1st instar nymph; 2nd: 2nd instar nymph; 3rd: 3rd instar nymph; 4th: 4th instar nymph. The gene expression levels at various developmental stages were normalized to that in the 1st instar nymph. Statistical analysis was performed by Tukey's HSD test after one-way ANOVA. Different letters above bars (means ± *SD*) indicate significant difference ($P < 0.05$).

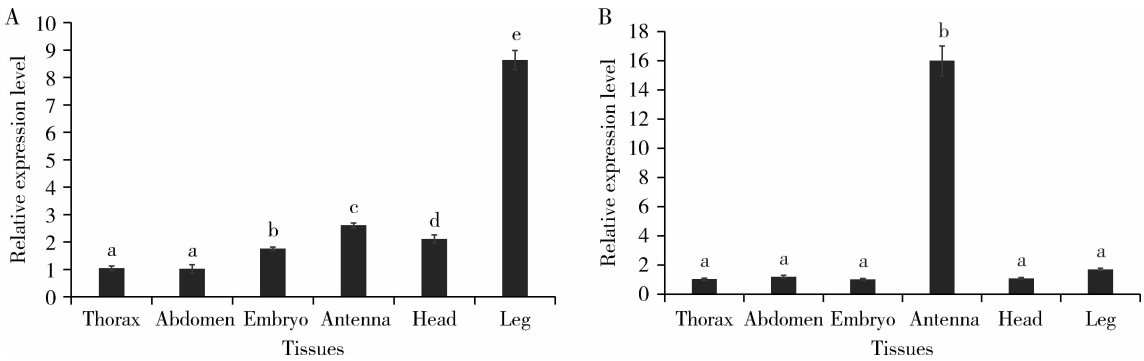

Fig. 3　Relative expression levels and tissue distribution of *LeryOBP*3 (A) and *LeryOBP*7 (B) in *Lipaphis erysimi* wingless adults
The gene expression levels in various adult tissues were normalized to that in thorax. Statistical analysis was performed by Tukey's HSD test after one-way analysis of variance (ANOVA). Different letters above bars (means ± *SD*) indicate significant difference ($P < 0.05$).

The aphids which feed exclusively on Brassicaceae plants have evolved a particular biochemical mechanism that degrades the plant glucosinolates into volatile compounds of isothiocyanates which acted as synergists of alarm pheromone (Jones *et al.*, 2001; Francis *et al.*, 2004; Blande *et al.*, 2007). The use of isothiocyanates could not only be directly toxic to some natural enemies of aphids but also serve as a feeding deterrent for non-Cruciferae feeding aphids to avoid potential competitions (Francis *et al.*, 2001; Vanhaelen *et al.*, 2001). However, how the isthiocyanates synergized the behavioral response of turnip aphid to EBF and the particular olfactory mechanism need to be studied further.

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萝卜蚜反-β-法尼烯结合蛋白基因的时空表达模式分析

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摘要：【目的】气味结合蛋白（odorant binding proteins，OBPs）有助于提高昆虫嗅觉系统的敏感性，并且在蚜虫报警信息素反-β-法尼烯[(*E*)-β-farnesene EBF]的通信过程中起重要作用。萝卜蚜 *Lipaphis erysimi* 是十字花科作物的主要害虫，为了减少蔬菜田间的农药使用，EBF 对蚜虫防治具有很大的潜力。然而，目前萝卜蚜对 EBF 反应的研究甚少。【方法】本研究利用 PCR 和 RACE 技术克隆并鉴定了 2 种公认的在萝卜蚜中对 EBF 具有高度亲和力的气味结合蛋白的基因 *LeryOBP*3 和 *LeryOBP*7；利用 RT-qPCR 检测了它们在萝卜蚜不同发育阶段和无翅成蚜不同组织中的表达谱。【结果】克隆获得的萝卜蚜 2 个基因序列 *LeryOBP*3（GenBank 登录号：KJ703012）和 *LeryOBP*7（GenBank 登录号：KJ703013）分别与豌豆蚜 *Acyrthosiphum pisum ApisOBP*3 和 *ApisOBP*7 序列对比有较高的氨基酸序列一致性（分别为 94% 和 88%），基因序列符合气味结合蛋白基因的典型特征。*LeryOBP*3 和 *LeryOBP*7 开放阅读框全长分别为 357 和 468 bp，分别编码 118 和 155 个氨基酸。发育表达谱表明，*LeryOBP*3 和 *LeryOBP*7 的表达量高峰出现于成虫期；组织表达谱表明，*LeryOBP*3 在成蚜足中表达量较高，而 *LeryOBP*7 主要表达于成蚜触角组织中。【结论】在萝卜蚜成虫触角中的 LeryOBP7 结合蛋白可能与其对 EBF 的响应有关。

关键词：萝卜蚜；报警信息素；气味结合蛋白；基因表达谱；实时荧光定量 PCR

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